Role of Chemokines and Formyl Peptides in Pneumococcal Pneumonia-Induced Monocyte/Macrophage Recruitment[1]

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Host-derived chemoattractant factors are suggested to play crucial roles in leukocyte recruitment elicited by inflammatory stimuli in vitro and in vivo. However, in the case of acute bacterial infections, pathogen-derived chemoattractant factors are also present, and it has not yet been clarified how cross-talk between chemoattractant receptors orchestrates diapedesis of leukocytes in this context of complex chemoattractant arrays. To investigate the role of chemokine (host-derived) and formyl peptide (pathogen-derived) chemoattractants in leukocyte extravasation in life-threatening infectious diseases, we used a mouse model of pneumococcal pneumonia. We found an increase in mRNA expression of eight chemokines (RANTES, macrophage-inflammatory protein (MIP)-1α, MIP-1β, MIP-2, IP-10, monocyte chemoattractant protein (MCP)-1, T cell activation 3, and KC) within the lungs during the course of infection. KC and MIP-2 protein expression closely preceded pulmonary neutrophil recruitment, whereas MCP-1 protein production coincided more closely than MIP-1α with the kinetics of macrophage infiltration. In situ hybridization of MCP-1 mRNA suggested that MCP-1 expression started at peribronchovascular regions and expanded to alveoli-facing epithelial cells and infiltrated macrophages. Interestingly, administration of a neutralizing Ab against MCP-1, RANTES, or MIP-1α alone did not prevent macrophage infiltration into infected alveoli, whereas combination of the three Abs significantly reduced macrophage infiltration without affecting neutrophil recruitment. The use of an antagonist to *N*-formyl peptides, *N-t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe, reduced both macrophages and neutrophils significantly. These data demonstrate that a complex chemokine network is activated in response to pulmonary pneumococcal infection, and also suggest an important role for fMLP receptor in monocyte/macrophage recruitment in that model. *The Journal of Immunology,* **2001, 166: 7353–7361.**

Effective host defense against lung bacterial infection is primarily dependent on rapid bacterial clearance mediated by macrophages and neutrophils. The recruitment of leukocytes at the site of infection involves the coordinated expression of leukocyte and vascular adhesion molecules such as integrins and selectins, as well as the establishment of gradients of chemotactic factors released from host cells (such as chemokines, leukotriene B_4, platelet activating factor, C5a complement component) or emanating directly from the invading pathogen (such as formyl peptides). Chemokines are a group of structurally and functionally related small proteins secreted by leukocytes and nonimmune cells, which are endowed with chemoattractant activity for particular leukocytes (1, 2). They are subdivided into four groups based on the position of their conserved cysteine residues (1, 3). The closely related C-X-C and C-C families have been increasingly recognized as important mediators in a variety of inflammatory disease states. C-X-C chemokines include murine (KC) and macrophage-inflammatory protein (MIP)[4]-2, which are predominantly stimulatory and chemotactic toward neutrophils, whereas CC chemokines including monocyte chemoattractant protein (MCP)-1, MIP-1α, and RANTES preferentially augment monocyte/macrophage and lymphocyte recruitment.

The recruitment of monocytes/macrophages at sites of inflammation generally occurs after neutrophil emigration in vivo, and roles for C-C chemokines have been explored in various models of lung inflammation. For example, MCP-1 appears to be involved in the emigration of monocytes/macrophages in lung allergic inflammation (4) and pulmonary *Cryptococcus neoformans* infection (5) but not in IgG immune complex acute lung injury (6). Neutralization of RANTES decreases macrophage infiltration to allergic inflammation (4) but not in IgG immune complex acute lung injury (6). MIP-1α is suggested to participate in the alveolar diapedesis of macrophage in pulmonary *C. neoformans* infection (7) but not in lung allergic inflammation (4). Although an increase in the expression of three C-C chemokines in lung is often observed in those lung infection/inflammation models, the above studies suggest that such accumulation of chemokines in lung may not necessarily be correlated with extravasation of macrophages in alveolar spaces. Thus, other hierarchical regulation may play a role for appropriate extravasation of macrophages in alveoli.

With their elegant in vitro works, the group of Butcher proposed that cross-talk between various chemoattractant receptors guides leukocytes to their destinations (8–10). Particularly interesting are cases of infections where pathogen-derived chemoattractant factors such as formyl peptides are present. Formyl peptides are

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[4] Abbreviations used in this paper: MIP, macrophage-inflammatory protein; MCP, monocyte chemoattractant protein; BAL, bronchoalveolar lavage; RPA, RNase protection assay; Boc-PLPLP, *N-t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe; TCA, T cell activation; MPO, myeloperoxidase; DIG, digoxigenin; ISH, in situ hybridization.

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cleavage products of bacterial or mitochondrial proteins and serve as powerful chemoattractants for both neutrophils and monocytes. Moreover, occupation of formyl peptide receptor with formyl peptides induces heterologous desensitization of receptors for other chemoattractant molecules, including chemokines (8, 11). Whether cross-talk exists between host- and pathogen-derived chemoattractants in in vivo infection models has not yet been established rigorously.

In streptococcal pneumonia, active monocyte/macrophage extravasation takes place in late stage of infection when pathogens proliferate (12). It has been suggested that such *Streptococcus pneumoniae* growth could result in sustained release of formyl peptides. Thus, it is likely that high levels of formyl peptide in infected lungs contribute to macrophage emigration in alveoli. Moreover, we have previously suggested that an early and sustained TNF-α/IL-1 production takes place in *S. pneumoniae* pneumonia (12). As it has been suggested by in vitro and in vivo studies that TNF-α and IL-1 induce chemokine production (13–15), we investigated in this study whether chemokines and formyl peptides are involved in monocyte/macrophage recruitment to alveolar spaces infected with *S. pneumoniae*.

We found mRNA expression of eight chemokines, RANTES, MIP-1α, MIP-1β, MIP-2, IFN-γ-inducible protein 10 (IP-10), MCP-1, T cell activation 3 (TCA)-3, and KC within the lungs during the course of murine pneumococcal pneumonia. KC and MIP-2 protein expression in lungs correlated relatively well with the kinetics of neutrophil infiltration. Sustained MCP-1 protein production coincided more closely than MIP-1α with the kinetics of monocyte/macrophage infiltration in alveoli. In situ hybridization (ISH) of MCP-1 mRNA suggested that MCP-1 expression starts at peribronchovascular regions and expands to alveoli-facing epithelial cells and infiltrated macrophages. Administration of a neutralizing Ab against MCP-1, MIP-1-α, or RANTES alone failed to reduce macrophage recruitment to infected alveoli, whereas combination of the three Abs reduced it by 33% ($p < 0.05$). Injection of an antagonist to *N*-formyl peptides, *N-t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe (Boc-PLPLP), reduced macrophage recruitment to infected alveoli by 42% ($p < 0.05$), suggesting an important role for bacterial formyl peptides as chemoattractants for macrophages during streptococcal pneumonia.

Materials and Methods

Reagents

Polyclonal goat anti-murine MCP-1, MIP-1α, KC, and MIP-2 Abs used in our ELISAs and in neutralization studies were purchased from R&D Systems (Minneapolis, MN). mAb to mouse MCP-1 used in neutralization studies was purchased from PharMingen (Mississauga, Ontario, Canada). The mouse rMCP-1, rMIP-1α, rKC, and rMIP-2 were purchased from R&D Systems. Boc-PLPLP was obtained from Sigma (Mississauga, Ontario, Canada).

Pneumococcal pneumonia model

A murine model of pneumococcal pneumonia has been developed in our laboratory (12). Briefly, lightly anesthetized female CD1 mice (18–20 g) received an inoculum of 10^7 CFU of *S. pneumoniae* in 50 μl of PBS applied at the tip of the nose and involuntarily inhaled. This inoculation induced typical pneumonia symptoms (12) and 100% mortality rate within 4 days. In this experiment, infected animals were sacrificed by decerebration (under anesthesia) at different time points after infection over a 3-day period. Blood, bronchoalveolar lavage (BAL) fluid, and lung tissue were sampled for assessment of leukocyte recruitment, chemokine mRNA expression, and protein level. For plasma sampling, blood was collected in heparinized tubes from the retro-orbital sinus of the left eye, immersed in ice, and centrifuged (1000 $\times$ *g* at 4°C for 15 min). BAL was performed as previously described (12). Briefly, the trachea was exposed and intubated with a catheter, then the lungs were washed two times with 1 ml PBS. BAL fluids were centrifuged at 550 $\times$ *g* for 10 min, and supernatants were stored at −70°C until evaluation of the chemokines by ELISAs. Pellets were resuspended in PBS for total BAL cell count with a hemacytometer and for differentiation of the cell populations with Diff-Quick-stained (Baxter, Quebec, Canada) cytospin preparations. Lungs were also taken for assessment of neutrophil infiltration and chemokine protein expression in tissue. First, the pulmonary vasculature was perfused with 10 ml PBS injected via the right ventricle of the heart, then lungs were homogenized in 50 mM sodium phosphate buffer containing 0.5% Triton X-100 for chemokine analysis or 0.5% hexadecyltrimethylammonium bromide for measuring myeloperoxidase (MPO) activity (pH 7.5). For chemokine analysis, homogenates were incubated on ice for 30 min, then centrifuged for 15 min at 4°C (3000 $\times$ *g*), and supernatants were stored at −70°C until measurements. Neutrophil infiltration in lung tissue was determined by MPO activity, as previously described (12). Briefly, homogenates were sonicated and centrifuged at 3000 $\times$ *g* in a microcentrifuge for 30 min at 4°C. The supernatants were then mixed 1:8 with assay buffer, and absorbance was read at 450 nm against a standard curve made with commercial MPO (Sigma).

Measurement of mRNA expression by Northern blots and RNase protection assay (RPA)

Whole lungs were harvested at specific times after inoculation with *S. pneumoniae*. They were immediately homogenized in 4 ml of TRIzol reagent (Life Technologies, Grand Island, NY) and stored at −70°C. Total cellular RNA was isolated from lung tissues by acid guanidinium thiocyanate-phenol-chloroform extraction according to the manufacturer's protocol. KC mRNA expression was determined by Northern blots. For each sample, 10 μg of RNA was separated in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (MANDEL Scientific, Quebec, Canada). Blots were then hybridized with a KC cDNA probe labeled by random priming using the T7 QuickPrime kit (Pharmacia Biotech, Uppsala, Sweden). Hybridized probe was visualized by autoradiography.

Chemokine mRNA expression was also determined by multiprobe RPA using the RiboQuant RPA kit (PharMingen) as recommended by the supplier. The specific chemokine bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The sample loading was normalized by the housekeeping genes L32 and GAPDH.

Determination of chemokine protein expression

Sandwich ELISAs were established for KC, MIP-2, MIP-1α, and MCP-1 to detect these chemokines in the supernatant of BAL fluid, in the supernatant of lung homogenates, and in plasma. Briefly, 96-well plates (Immunoplate; Nunc, Naperville, IL) were coated with anti-MCP-1, anti-MIP-2, anti-MIP-1α, or anti-KC in 100 μl/well of PBS, pH 7.4. Plates were incubated for 16 h at 4°C. Each plate was blocked with 5% BSA-PBS, and an appropriately diluted sample (100 μl) was incubated for 2 h at room temperature. The biotinylated polyclonal goat anti-chemokine Ab (100 μl/well) was added and incubated for 1 h in 2% BSA-PBS. Bound biotinylated anti-chemokine Ab was detected by streptavidin-peroxidase conjugate (Research Diagnostics, Flanders, NJ). The limit of detection for each chemokine was MCP-1, 20–500 pg/ml; MIP-1α, 20–500 pg/ml; KC, 20–1000 pg/ml; and MIP-2, 20–500 pg/ml.

Preparation of murine JE (MCP-1) digoxigenin (DIG) cRNA probes

Murine JE (MCP-1) DIG-cRNA probes were synthesized by in vitro transcription of a JE cDNA fragment of 600 bp subcloned into the pGEM-1 vector. After linearization of the vector with *Hin*dIII, the antisense probe was transcribed according to the manufacturer's protocol using T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany) and nucleoside triphosphates including digoxigenin-uridine triphosphate (Dig-11-dUTP; Boehringer); the control sense probe was prepared by linearization with *Pvu*II and transcription with Sp6 RNA polymerase (Boehringer Mannheim).

ISH for MCP-1

The ISH protocol was established from a combination of published procedures (16, 17). All reactions were conducted with RNase-free materials and solutions. Fresh left lungs were perfused and fixed overnight at 4°C in 0.1 M phosphate-buffered 4% paraformaldehyde. Tissues were then dehydrated in solvent and embedded in paraffin. Five-micrometer sections were collected on positively charged slides (BioGenex Laboratories, San Ramon, CA) and dried overnight at 37°C.

Tissue sections were incubated in Tissue Clear III (Fisher Scientific, Pittsburgh, PA) and rehydrated by passage through graded ethanol and

RNase-free water, followed by enzymatic treatment with 10 μg/ml proteinase K (Boehringer Mannheim) for 15 min at 37°C. Sections were then acetylated with freshly prepared 0.25% acetic anhydride, 0.1 M triethanolamine, pH 8.0, for 10 min.

Lung tissue sections were then hybridized overnight at 44°C with 25 μl heat-denatured antisense or sense DIG-MCP-1 cRNA probes at a concentration of 100 ng/ml in hybridization solution (50% formamide, 4× SSC, 1× Denhardt's solution, 1 mg/ml salmon sperm, and 10× dextran sulfate). Hybridization reactions were detected by immunostaining with alkaline phosphatase-conjugated anti-DIG Abs (Boehringer Mannheim). Nonspecific staining was blocked by incubation for 30 min with 1% lamb serum in TBS buffer containing 0.1% Triton X-100. After an incubation of 1 h with anti-DIG Ab diluted in blocking buffer, the hybridization signal was visualized with the substrates nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt. Levamisole (Sigma) was added to the reaction to inhibit endogenous alkaline phosphatase. Sections were mounted with AquaPerm (Fisher Scientific).

Passive immunization with neutralizing Abs against chemokines

Groups of infected mice received daily i.v. doses of 20 μg/mouse of neutralizing polyclonal Ab against MIP-1α or 5 μg/mouse of mAb against MCP-1 based on reported neutralizing efficacy in animal models (4, 18), or 1 μg/mouse of polyclonal Ab against RANTES based on the recommended dose of manufacturer. A last group received a mixture of 20, 1, and 5 μg/mouse of anti-MIP-1α, anti-RANTES, and anti-MCP-1, respectively. For each Ab, the first administration was performed 30 min before the inhalation of *S. pneumoniae*, and subsequent doses were repeated on days 1 and 2. Control mice ("preimmune") received normal goat IgG for anti-MIP-1α and for anti-RANTES, and normal hamster IgG for anti-MCP-1 (Jackson ImmunoResearch Laboratories, West Grove, PA). Mice were then killed 6 h after final administration of Abs (54-h postinfection) on day 2, and BALs were collected to analyze leukocyte counts.

Inhibition of fMLP activity in vivo

The fMLP antagonist Boc-PLPLP (Sigma) (19) was injected i.v. at a concentration of 2×10^{-4} M, 0.1 ml/mouse, 48 h after infection with *S. pneumoniae*. Animals were sacrificed 6 h after injection of the drug. This product, when injected at this dose to uninfected mice, did not induce leukopenia.

Statistical analysis

All statistical analyses were performed using a computer package (Statview+SE Software; Abacus Concepts, Berkeley, CA). The significance between groups was statistically evaluated using a one-way ANOVA test, followed by the *t* test with Fisher's corrections.

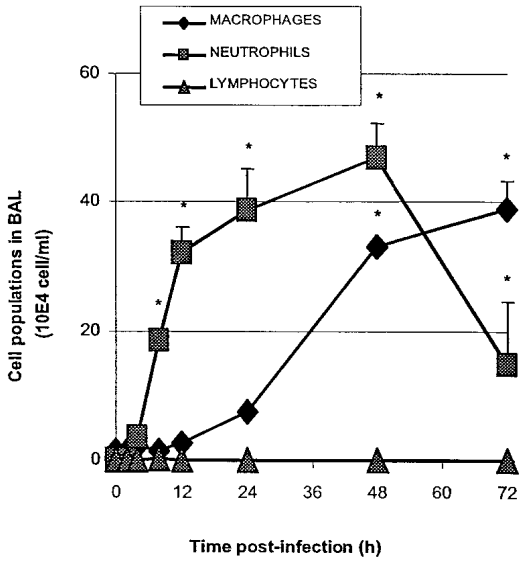

FIGURE 1. Recruitment of inflammatory cells (means ± SEM) in BAL fluid as a function of time after infection of CD1 mice with 10^7 CFU of *S. pneumoniae*. *, $p < 0.05$ vs uninfected mice (0 h).

Results

The mouse model of streptococcal pneumonia

In our streptococcal pneumonia model, neutrophils started to appear in alveoli (where the infection occurred) 4 h after inoculation with *S. pneumoniae*, as we published before (12). The number of neutrophils recruited in alveoli sharply increased at 12 h, maintained high levels until they peaked at 48 h, then were reduced at the point of death 72–96 h after infection (Fig. 1). A small number (1.4×10^4 cells/ml) of resident alveolar macrophages were present in alveoli before the infection, and newly recruited macrophages became evident into alveoli at 48 h postinfection. Lymphocytes and eosinophils remained essentially absent in BAL over the duration of the experiment.

The number of leukocytes recruited in alveoli correlated well with the histological profile of leukocytes recruited in lung tissue, with the exception that significant neutrophil accumulation (MPO activity) was detectable as early as 4 h in tissues, and exudation to alveolar spaces occurred only after 8 h (data not shown). These data corroborated our previously reported observations (12).

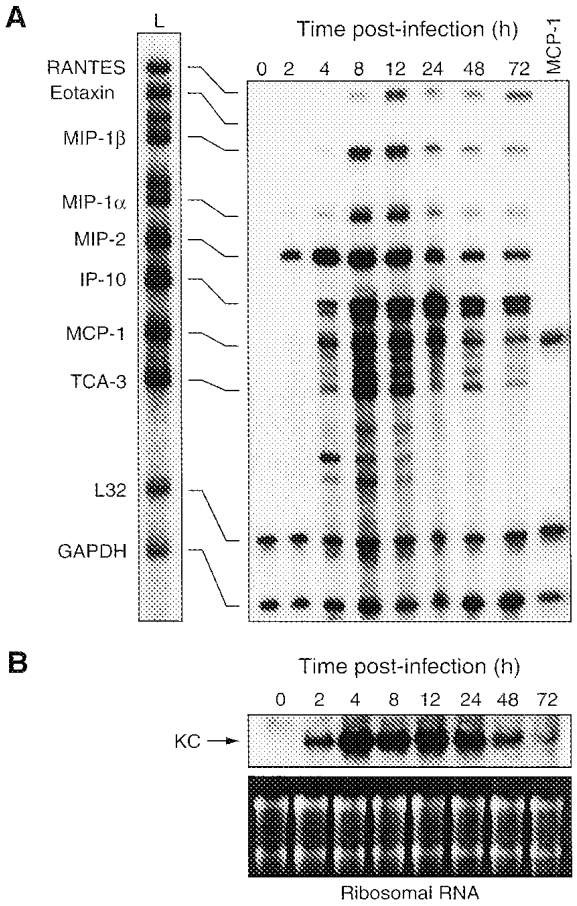

FIGURE 2. Chemokine expression in whole lung during pneumococcal pneumonia. Total RNA was extracted from lung homogenates obtained at 0, 2, 4, 8, 12, 24, 48, and 72 h after infection. It was hybridized to PharMingen CK5 probe template set (*A*) and to KC probe (*B*). Each band represents mRNA from three mice at the time points indicated. *A*, RPAs. RNase-protected fragments were analyzed on polyacrylamide gels that were subsequently exposed to x-ray film to visualize protected bands. *Far left lane*, Undigested probe template set that served as a size reference to which the protected fragments were compared. *Far right lane*, Total RNA from 8 h was hybridized with MCP-1 probe template to confirm position of MCP-1 band. *B (top)*, Northern blot analysis for KC mRNA; *bottom*, equal loading was confirmed by ethidium bromide staining of 18S and 28S bands.

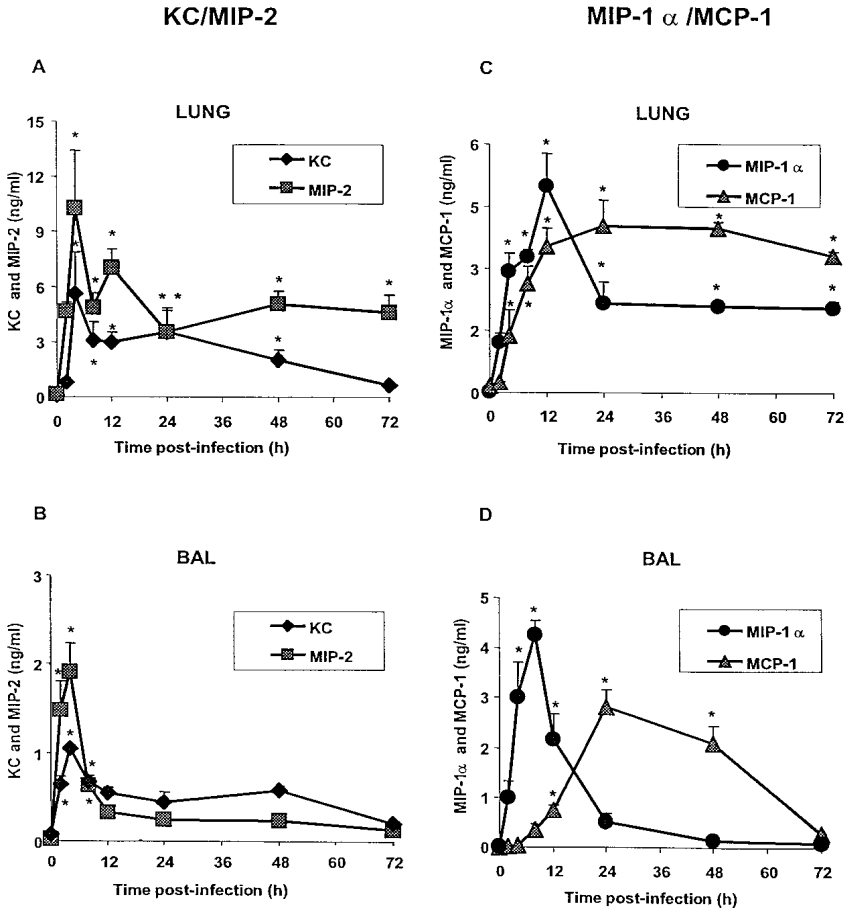

FIGURE 3. CXC (KC and MIP-2) protein production (*A* and *B*), and CC (MIP-1α and MCP-1) protein production (*C* and *D*) in lung homogenates (*A* and *C*) and BAL fluid (*B* and *D*) of CD1 mice as a function of time after intranasal administration of 10^7 CFU *S. pneumoniae*. *, $p < 0.05$ vs uninfected mice (0 h). Data are means ± SEM of four mice per group per time point.

Chemokine production

To investigate whether biosynthesis of C-X-C chemokines (chemoattractants for neutrophils) and C-C chemokines (chemoattractants for monocytes/macrophages) is up-regulated during murine pneumococcal pneumonia, chemokine mRNA expression in whole lungs was first analyzed at various times (0–72 h) by Northern blots and RPA. The RPA had the capacity to simultaneously detect several chemokine mRNA species in a single sample of RNA. The chemokine protein production in whole lungs and their release in alveoli (BAL) were also estimated by specific sandwich ELISAs. Northern and RPA analysis (Fig. 2) demonstrated that substantial amounts of C-X-C chemokine MIP-2 (Fig. 2*A*) and KC (Fig. 2*B*) mRNA were present in lungs as early as 2 h after infection. They peaked at 4 h, and appeared to plateau from 8 to 24 h after the infection. Thereafter, from 48 to 72 h, mRNA levels gradually decreased for KC but persisted to a certain extent for MIP-2. KC and MIP-2 proteins were also detected at 2 h and peaked at 4 h in both lungs and BAL fluid (Fig. 3, *A* and *B*). The same pattern as for mRNA detection was observed, as KC decreased gradually over time to reach basic level at 72 h, whereas MIP-2 in lungs persisted at a significant level until death of the animals. Therefore, KC and MIP-2 protein levels were consistent with their respective mRNA expression. Moreover, the peak concentrations of KC and MIP-2 proteins coincided with the initiation of neutrophil infiltration into lung interstitium (MPO activity) and alveoli (cell counts), but only the persistence of MIP-2 coincided with the sustained recruitment of neutrophils late after infection (Figs. 1 and 3*A*). Interestingly, KC was detected in plasma as early as 4 h after infection (Fig. 4) and remained high until 72 h, whereas MIP-2 appeared only after 48 h. As for IP-10, the mRNA expression of this C-X-C chemokine occurred later than that of KC or MIP-2, but high levels were seen from 8 to 24 h and were maintained to some extent until 72 h (Fig. 2).

The mRNA expression of various C-C chemokines, MIP-1α, MIP-1β, MCP-1, TCA-3, and RANTES, which was undetectable in uninfected lungs, increased as early as 4 and 8 h after infection (Fig. 2). (In fact, in the multiple probes present in the RPA, only eotaxin was not expressed at any time during the experiment). However, the kinetics and/or magnitude of expression of these mRNA were distinct for each C-C chemokine, suggesting their involvement at different time points during pneumonia.

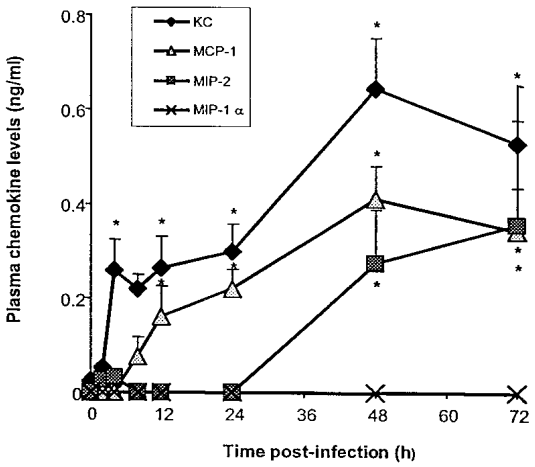

FIGURE 4. Mean (SEM) levels of KC, MIP-2, MCP-1, and MIP-1α in plasma of mice infected with *S. pneumoniae*. *, $p < 0.05$ vs uninfected mice (0 h). Data are means ± SEM of $n = 4$ mice per group.

There was a marked difference in the time-dependent expression of mRNA and protein between MCP-1 and MIP-1α during the course of infection. At the protein level, MIP-1α was the first to be recovered in BAL, with a significant increase from 2 to 8 h after infection (Fig. 3*D*) when there was no significant macrophage recruitment (Fig. 1). Despite the fact that evolution of the infection accelerated macrophage recruitment into alveolar spaces 48 h after infection, levels of MIP-1α in BAL were reduced significantly after 24 h (Fig. 3*D*). However, levels of MIP-1α in total lungs remained detectable throughout infection (Fig. 3*C*). MCP-1 protein production in the lungs became evident at 4 h and reached a plateau at 12 h that was sustained up to 72 h when animals started to die (Fig. 3*C*), indicating that the kinetics of MCP-1 expression in lungs preceded actual macrophage recruitment. In BAL, MCP-1 protein secretion to alveoli was delayed in the first 12 h but strongly increased between 24 and 48 h when macrophage recruitment into alveoli became evident (Figs. 1 and 3*D*). MCP-1 proteins in BAL decreased to background levels thereafter despite continuous biosynthesis in lung tissues. These data demonstrate that the kinetics of MCP-1 protein in alveolar spaces correlates better than that of MIP-1α to the emigration of macrophages to alveoli, which took place within 48 h after infection (Figs. 1 and 3, *C* and *D*). Finally, MCP-1 was recovered in plasma from 8 to 72 h, whereas MIP-1α remained essentially absent from bloodstream throughout the evolution of pneumonia (Fig. 4).

ISH for MCP-1

We had detected an early and sustained MCP-1 release in lungs and alveolar spaces, suggesting that MCP-1 might form a gradient to induce monocyte/macrophage recruitment to infected alveoli. Thus, we investigated the distribution of MCP-1 mRNA by ISH using digoxigenin-conjugated antisense probes on paraffin sections of mouse lung tissues infected with *S. pneumoniae*. In normal lung tissue, no MCP-1 mRNA was detected (Fig. 5*A*). In the first 24 h

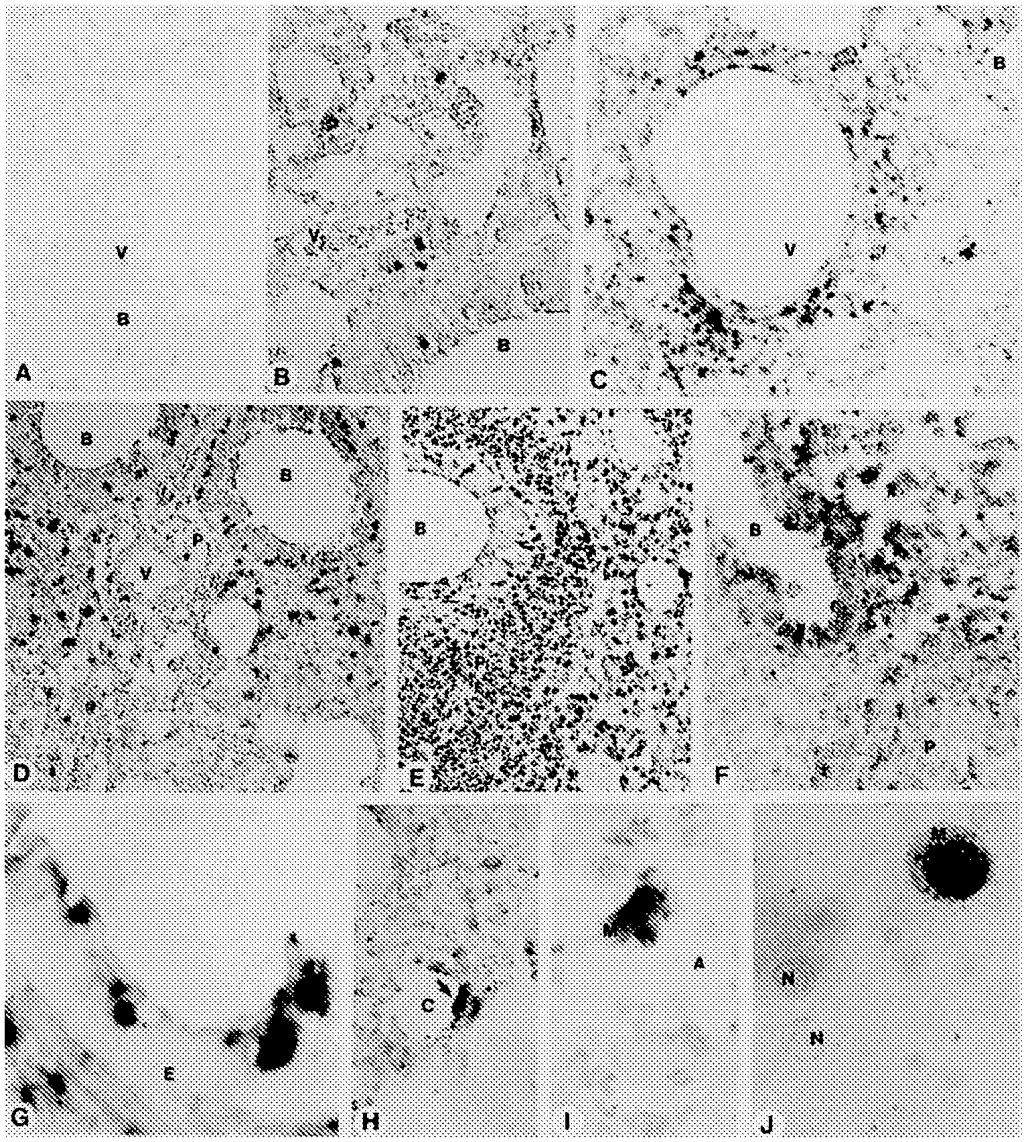

FIGURE 5. ISH of MCP-1 mRNA expression in lung tissues of mice infected with *S. pneumoniae*. Normal lung tissue was negative for MCP-1 mRNA (*A*). From 2 to 12 h after infection (*B*, 2 h; *C*, 4 h; and *D*, 12 h), MCP-1 mRNA expression gradually expanded from peribronchovascular areas to surrounding lung tissues. At 48 h (*E*), lung tissue and leukocytes in perivascular spaces extensively expressed MCP-1 mRNA. At 72 h (*F*), lung tissue still stained positive for MCP-1 mRNA, but low levels were observed in perivascular spaces. *G*, Bronchiolar epithelial cell. *H*, Capillary endothelial cell. *I*, Alveolar macrophage. *J*, Macrophage and neutrophils in BAL. Magnifications: *A*, ×70; *B* and *H*, ×280; *C*, *D*, and *E*, ×140; *F*, ×225; and *G*, *I*, and *J*, ×740. A, Alveolus; arrow, capillary endothelial cell; B, bronchiole; C, capillary; E, bronchiolar epithelial cell; M, macrophage; N, neutrophil; P, perivascular space; V, blood vessel.

Table I. *Effect of administration of single neutralizing Abs against specific C-C chemokines on leukocyte recruitment in BAL fluid of mice 2 days after infection with* S. pneumoniae[a]

Treatment of Mice	No. of Mice	No. of Monocytes/ Macrophages (10^4)
Control IgG + *S. pneumoniae*	5	4.3 ± 0.3
Anti-MCP-1 + *S. pneumoniae*	6	5.7 ± 1.2
Control IgG + *S. pneumoniae*	6	7.6 ± 2.6
Anti-MIP-1α + *S. pneumoniae*	6	6.3 ± 1.1
Control IgG + *S. pneumoniae*	6	4.0 ± 0.7
Anti-RANTES + *S. pneumoniae*	5	5.9 ± 0.8

[a] Results are expressed as means ± SEM. Mice were administered three doses of anti-MCP-1, anti-MIP-1α, or anti-RANTES at 30 min before infection, 24 h and 48 h after infection. The time of sacrifice was 6 h after the final treatment (54 h post-infection).

after infection, MCP-1 mRNA was mainly detected in peribronchovascular areas (Fig. 5, *B–D*). Over that period, some bronchiolar epithelial cells (Fig. 5*G*), capillary endothelial cells (Fig. 5*H*), and alveolar macrophages (Fig. 5*I*) localized in these areas expressed MCP-1 mRNA. Then, as infection progressed, labeled mRNA expanded from large peribronchovascular sites to surrounding tissues and alveolo-capillary spaces where endothelial cells, alveolar macrophages, and numerous recruited leukocytes were the main expressing cells (Fig. 5*E*). In fact, maximal labeling in the lung was noted at 48 h postinfection (Fig. 5*E*), when strong recruitment of macrophages occurred (Fig. 1). At 72 h, when active emigration of macrophages to alveoli was observed, MCP-1 mRNA expression declined in peribronchovascular spaces but remained present at various pulmonary sites (Fig. 5*F*). Control ISH sections exposed to sense probe or without any probe were unreactive, thereby confirming specificity of the detection signals (data not shown).

As we had observed that only a few leukocytes expressed MCP-1 mRNA in the perivascular spaces at early time points (Fig. 5*D*) while most of the recruited leukocytes expressed MCP-1 mRNA at later time points (Fig. 5*E*) in the whole lung surface, we performed an additional assay to determine which type of infiltrating cells expressed MCP-1 in our model. ISH was made on cells obtained from BAL fluid 48 h postinfection, when neutrophil and macrophage recruitment was active. Interestingly, among the leukocytes present in BAL, expression of MCP-1 mRNA was observed in macrophages but not in neutrophils (Fig. 5*J*).

Treatment	No. of mice	Monocytes/macrophages ($\times 10^4$)
Control IgG	12	4.2 ± 0.3
anti-MCP-1/MIP-1α/RANTES	12	2.8 ± 0.5*

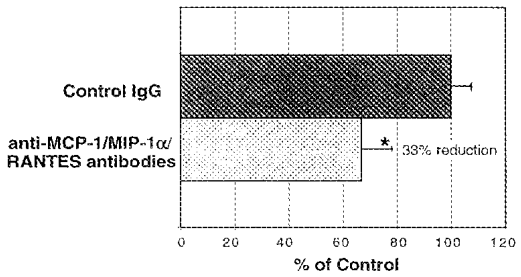

FIGURE 6. Monocytes/macrophages in BAL fluid of mice infected with 10^7 CFU of *S. pneumoniae* and treated with three i.v. injections of a combination of Abs against MCP-1, MIP-1α, and RANTES. Times of injection were 30 min before infection as well as 24 and 48 h after infection. Animals were sacrificed 6 h after the last 48-h injection of Abs. *, $p < 0.05$ compared with untreated infected mice.

Treatment	No. of mice	Monocytes/macrophages ($\times 10^4$)
Control	10	8.8 ± 1.3
Boc-PLPLP	9	5.5 ± 0.7*

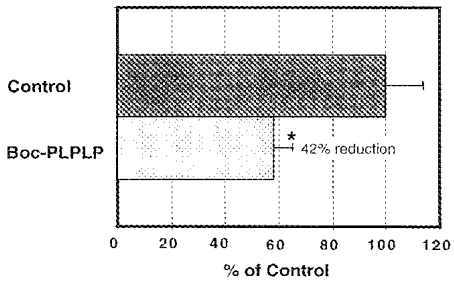

FIGURE 7. Monocytes/macrophages in BAL fluid of mice infected with 10^7 CFU of *S. pneumoniae* and treated 48 h later with one i.v. dose of 10^{-4} M Boc-PLPLP. Animals were sacrificed 6 h after injection of Boc-PLPLP. *, $p < 0.05$ compared with untreated infected mice.

Influence of neutralizing Abs specific to C-C chemokines

The above results suggested that sustained MCP-1 expression and late monocyte/macrophage recruitment characterized our lethal pneumonia model. To further evaluate the specific contribution of C-C chemokines (MCP-1, MIP-1α, and RANTES) to monocyte/macrophage recruitment, chemokine-inhibition experiments were performed using specific neutralizing Abs that have been characterized extensively in vitro and in vivo. Infected mice were injected with three doses of anti-MCP-1, anti-MIP-1α, or anti-RANTES alone, or a combination of the three neutralizing Abs 30 min before infection, as well as 24 and 48 h after infection. The extent of monocyte/macrophage emigration to alveoli (BAL fluid) was studied 6 h after final administration of Abs (active macrophage emigration to alveoli is observed at 48 h postinfection). Control mice received control preimmune IgG.

As shown in Table I, none of the neutralizing Abs given alone could reduce monocyte/macrophage recruitment in BAL fluid at 54 h postinfection. Neutrophil counts were not reduced as well (data not shown). In contrast to administration of Ab against individual chemokine, passive immunization with the combination of Abs against three C-C chemokines (MCP-1, MIP-1α, and RANTES) reduced the number of monocytes/macrophages emigrated to alveoli by 33% ($p < 0.05$) compared with the amount recovered in mice administered the control IgG (Fig. 6). Administration of the combined Abs slightly reduced neutrophil counts ($6.4 \pm 1.1 \times 10^4$ cells/ml for controls vs $4.9 \pm 0.6 \times 10^4$ cells/ml for treated mice), which was not statistically significant. Those data suggest that three major C-C chemokines participate in monocyte/macrophage recruitment to infected alveoli, but neutralization of a single chemokine among those three does not affect monocyte extravasation to alveoli.

Influence of formyl peptide receptor antagonist Boc-PLPLP on macrophage emigration to infected alveoli

We further evaluated the contribution of chemotactic formyl peptides derived from bacteria (such as fMLP) in our model of pneumonia. Blocking experiments were performed using an antagonist of formyl peptide receptor, Boc-PLPLP. Mice were injected with Boc-PLPLP 48 h after infection, and the amount of leukocytes

emigrated to infected alveoli was analyzed 6 h later. Fig. 7 shows a significant ($p < 0.05$) 42% reduction in monocyte/macrophage counts recovered in BAL fluid of mice treated with the antagonist as compared with the untreated infected group. Emigration of neutrophils in infected alveoli of mice treated with Boc-PLPLP also significantly ($p < 0.05$) decreased ($5.9 \pm 1.4 \times 10^4$ neutrophils/ml) compared with infected controls receiving placebo ($9.6 \pm 1.4 \times 10^4$ neutrophils/ml). Together, these data suggest that formyl peptides contribute to extravasation of both macrophages/monocytes and neutrophils to infected alveoli during pneumococcal pneumonia.

Discussion

The sequential recruitment of leukocytes toward sites of infection or inflammation is the most fundamental process of innate immunity. Over the last decade, many papers have suggested that involvement of specific C-C chemokines to monocyte/macrophage recruitment to inflamed lungs in various pulmonary diseases is dependent on the nature of the inflammatory Ag, although biosynthesis of various C-C chemokines was reported in most cases (4–7, 20–24). However, it has not been clarified yet how leukocytes interpret apparently conflicting chemoattractant stimuli in vivo. In the case of bacterial infections, large amounts of potent chemoattractant formyl peptides produced by bacteria are expected to be chronically present. As occupation of formyl peptide receptor by formyl peptides induces desensitization of receptors for host-derived leukocyte attractants (8, 11), progression of infection might affect leukocyte recruitment in a different manner than it is orchestrated in noninfectious acute lung injury.

In this experiment, pneumococcal pneumonia induced a rapid, time-dependent accumulation of neutrophils and monocytes/macrophages into the lungs. In our murine model of pneumonia, sustained neutrophil recruitment was observed from 4 h after infection until death, and it was followed by macrophage exudation that was significant from 48 h postinfection. Therefore, we consider this pneumonia model as an interesting in vivo approach to investigate how macrophages emigrate in the presence of multiple conflicting chemoattractant stimuli. In this study, our data demonstrate that biosynthesis and release of C-C chemokines, MCP-1, MIP-1α, MIP-1β, TCA-3, and RANTES, was up-regulated. Although MCP-1 expression in alveolar spaces relatively well paralleled the accumulation of macrophages in alveoli among the C-C chemokines, passive immunization with anti-MCP-1 alone did not significantly affect macrophage and neutrophil extravasation at a time when active monocyte/macrophage emigration and MCP-1 release were evident (48 h postinfection). Although passive immunization with Abs against MCP-1, MIP-1α, or RANTES alone could not affect monocyte/macrophage emigration to alveoli, administration of a combination of the three Abs against MCP-1, MIP-1α, and RANTES did reduce monocyte/macrophage recruitment significantly. Therefore, those three chemokines apparently participate in extravasation of monocytes/macrophages in a complementary manner, whereas each chemokine alone can replace the others to some degree. Administration of the formyl peptide antagonist Boc-PLPLP significantly reduced the emigration of both macrophages and neutrophils, suggesting that bacterial-derived formyl peptides also participate in the exudation process of macrophages in the late stage of pneumococcal pneumonia.

Infection with *S. pneumoniae* led to the rapid, coordinated, time-dependent expression of MIP-2 and KC chemokines in lung tissue and in BAL fluid. Although MIP-2 was secreted in larger amounts than KC, both pulmonary profiles for these C-X-C chemokines were quite similar and peaked closely before a strong neutrophil recruitment occurred in tissue and alveoli. This C-X-C chemokine kinetics suggests that MIP-2 and KC might be involved in neutrophil recruitment. Although this correlation is consistent with other lung infection models where overproduction or inhibition of KC or MIP-2 were respectively associated with increased (25) or attenuated (23, 24, 26, 27) neutrophil accumulation in alveoli, the exact roles for KC and MIP-2 in neutrophil recruitment in pneumococcal pneumonia remain to be clarified. As those C-X-C chemokine protein levels increase in blood in the late stage of infection, it is possible that the appropriate chemotactic gradient of C-X-C chemokines to attract neutrophils may not be formed in the late stage. Lack of appropriate chemotactic gradient by a particular chemokine could lead to the failure of leukocyte extravasation toward that chemokine. For example, it has been demonstrated that MCP-1-transgenic mice that express high levels of MCP-1 in blood and in a variety of organs do not display monocyte infiltration (28). Series of works by Doerschuk et al. (29–32) clearly demonstrate that in the case of pneumococcal pneumonia, a significant proportion of neutrophils emigrates in the alveolar spaces independently of two classical adhesion molecules expressed in neutrophils, β_2-integrins, and selectins. As those adhesion molecules participate dominantly in Gram-negative bacteria-induced acute lung infection and injury, pulmonary infection with *S. pneumoniae* seems to induce an alternative or "nonclassical" neutrophil diapedesis pathway. Therefore, it is possible that not only general C-X-C chemokines but also other classical chemoattractants are involved in the diapedesis of neutrophils in pneumococcal pneumonia, and this should be further investigated.

In our model, maximal monocyte/macrophage recruitment occurred between days 2 and 4 after infection. The mechanisms that govern specific monocyte/macrophage recruitment to alveoli are poorly understood. Previous investigations in other models of inflammation have suggested that monocyte recruitment depends upon both the activation of endothelium and the generation of monocyte chemoattractants (33). Yet this study demonstrated that a large array of C-C chemokines are expressed during *S. pneumoniae* infection, including MCP-1, MIP-1α, MIP-1β, RANTES, and TCA-3, whose mRNA expressions were detected in the lung as soon as 4 h or 8 h after infection. Although eotaxin has already been suggested to be a colony-stimulating factor for both granulocytes and macrophages in lung allergic inflammation (34), its main target cells are the eosinophils, which were not recruited in our model, and which also coincided with the absence of eotaxin mRNA expression in lung tissues. Interestingly, despite the fact that various C-C chemokines known to display overlapping activity for lymphocyte and monocyte recruitment were expressed in our model, very few, if any, lymphocytes emigrated into lung tissues. It is possible that recruitment and/or retention of lymphocytes in *S. pneumoniae*-infected alveoli need additional factors besides C-C chemokines. Alternatively, a chemoattractant gradient by formyl peptides that might not be chemotactic toward lymphocytes could play roles in leukocyte diapedesis in pneumococcal pneumonia.

Interestingly, the MCP-1 and MIP-1α protein kinetics were quite different during the evolution of pneumonia. MIP-1α secretion into infected alveoli peaked early (within 12 h after infection) and sharply decreased thereafter. As diapedesis of macrophages started 48 h after infection, it is unlikely that MIP-1α participated directly in the recruitment of macrophages to alveoli. This is also suggested by our experiment with anti-MIP-1α Ab, as neutralization of MIP-1α did not result in the reduction of macrophage recruitment in alveoli. In contrast to alveolar sites, a significant amount of MIP-1α protein was expressed in total lung tissue throughout infection, with no level of MIP-1α detected in plasma at any time. Thus, it is possible that MIP-1α might be involved in macrophage recruitment (or activation) into pulmonary interstitium.

The different patterns of MIP-1α and MCP-1 secretion that we observed in alveolar air spaces and blood suggest that expression of these two C-C chemokines might be mediated by disparate regulatory pathways, that these chemokines might be elaborated by mutually exclusive cells and/or tissues, and that they may serve nonoverlapping functions in modulating pulmonary host defenses. In fact, MCP-1 protein production in lungs and its accumulation in alveoli preceded and correlated more closely to the monocyte/macrophage infiltration in lungs than MIP-1α did. This sustained release of MCP-1 with live pneumococci is consistent with the same observation by Huffnagle et al. (5) in a mouse model of pulmonary *C. neoformans* infection. Although (among the C-C chemokines examined in this study) MCP-1 expression showed the closest correlation to the kinetics of pulmonary macrophage recruitment, the production of MCP-1 in lung and alveoli preceded macrophage recruitment in alveoli infected with *S. pneumoniae* (the time lag was between 24 and 36 h). This delay is in contrast to the *C. neoformans* infection where exact correlation between macrophage infiltration and MCP-1 accumulation in alveoli was observed (5). It is not entirely clear why such early C-C chemokine production in our model fails to induce immediate infiltration of macrophages. It is possible that monocytes/macrophages have been desensitized to C-C chemokines in the early stage of infection or that additional signals are necessary for emigration and differentiation/activation of these cells.

To better identify cells that synthesize MCP-1 and to follow the chemotactic gradient that is formed after pneumococcal infection, ISH was performed on lung tissue sections. Our results demonstrate that a variety of immune and nonimmune cells do synthesize MCP-1 mRNA in murine pneumococcal pneumonia. Endothelial cells in the peribronchovascular regions were among the main MCP-1 mRNA-expressing cells early after infection while labeling expanded to epithelial cells and monocytes/macrophages disseminated throughout the inflamed lungs at later stages of infection, suggesting that biosynthesis of MCP-1 is not exclusively limited to a particular cell type but rather is up-regulated in a synchronized manner that might be important for appropriate presentation of chemokines toward leukocytes. The difference in the profile of MCP-1 protein levels in lung tissue and BAL fluid determined by ELISA also suggests the existence of a gradient of chemokines to a certain degree. As macrophages that emigrate toward infected alveoli synthesize MCP-1, macrophages themselves are likely to participate in the establishment of such gradient. Although the complex events that initiated cell recruitment cannot fully be delineated from the present experiment, it is possible that bacterial chemotactic components or signals may have diffused freely from alveoli to surrounding tissues to blood vessels, as will be discussed later.

Although recruited monocytes/macrophages were able to express chemokines once at the site of infection, MCP-1 mRNA was not expressed by neutrophils in our pneumococcal pneumonia model. Papers regarding the ability of neutrophils to synthesize MCP-1 in vivo are controversial (35–37). Certain rat models of chronic bleomycin-induced lung fibrosis and chronic collagen-induced arthritis show MCP-1 production by infiltrated neutrophils (35, 36), whereas MCP-1 synthesis is not readily detectable in exudated neutrophils in acute skin or peritoneal inflammation (37). Therefore, the production of MCP-1 may be strictly regulated in neutrophils, depending on the type of inflammation and whether it is acute or chronic.

In our pneumococcal pneumonia model, neutralization of single C-C chemokine MCP-1, MIP-1α, or RANTES didn't enable us to confirm strict dependence of monocytes/macrophages on any of these three chemokines for emigration to infected alveoli. Significant reduction occurred only with neutralization of the three chemokines using a combination of appropriate Abs. In contrast, our data with Boc-PLPLP as an antagonist to formyl peptides demonstrated that *N*-formylated peptides released by pneumococci strongly contribute to chemotaxis of both neutrophils and macrophages in *S. pneumoniae*-infected alveoli. These small by-products of bacterial protein catabolism can diffuse freely within intercellular spaces (38) and are classical chemoattractants for neutrophils and macrophages. It has been shown that formyl peptides can be recovered in pneumococcal culture filtrates (39). Thus, our data suggest that chemoattractant bacteria-derived formyl peptides and host-derived chemokines were involved in the emigration of macrophages into alveoli in the late stage of pulmonary infection with *S. pneumoniae*. It is also possible that formyl peptides abrogated the host response to C-C chemokines to a certain extent in our model, as it has been demonstrated that preincubation of neutrophils or macrophages with formyl peptide inhibits their ability to respond to IL-8, or down-regulates CCR5, the receptor for MIP-1α and RANTES (8, 40). Interestingly, Campbell et al. (8) suggest that this preincubation with formyl peptide does not inhibit responsiveness to formyl peptide itself and proposed that chemoattractants emanating directly from a target of phagocytosis such as formyl peptide, would override signals generated from the surrounding host tissues such as chemokines. It has been suggested that fMLP and C5a receptors are the only receptors known to mediate heterologous desensitization. None of the tested chemokine or lipid attractant receptors examined to date appear to support uncoupling of heterologous responses (10). In our model, high pulmonary C-C chemokine (MCP-1, MIP-1α, and RANTES) levels were seen very early after infection, and macrophage recruitment was initiated relatively late, at a time when active bacterial growth prevails in inflamed lungs. Therefore, it is possible that, by the time peripheral monocytes started to migrate, their C-C chemokine receptors were partly insensitive to the chemoattractant gradient formed by C-C chemokines.

In conclusion, our study suggests that pneumococcus-derived formyl peptides participate as chemoattractants in neutrophil and macrophage infiltration to the alveoli observed in late stage of pneumococcal pneumonia, and that formyl peptide activity together with preexisting chemoattractant signals formed by chemokines actually guide monocytes/macrophages to the appropriate site of infection. Additional in vivo and in vitro studies are necessary to further delineate the respective roles of fMLP and of C-X-C and C-C chemokines in community-acquired and nosocomial pneumonia.

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